Pergamon

PII: S0031-9422(97)00442-1

Phytochemistry, Vol. 47, No. 4, pp. 521–526, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

COTTON VOLATILES SYNTHESIZED AND RELEASED DISTAL TO THE SITE OF INSECT DAMAGE*

P. W. PARÉ and J. H. TUMLINSON†

Center for Medical, Agricultural, and Veterinary Entomology, United States Department of Agriculture, Agricultural Research Service, 1700 SW 23rd Drive; Gainesville, Florida 32608, U.S.A.

(*Received* 4 *April* 1997)

Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; volatiles; semiochemical; plant–insect interactions; systemic response.

Abstract—Cotton plants (*Gossypium hirsutum*) damaged by herbivorous insects release volatile compounds that act as chemical signals to attract natural enemies of the herbivore to the damaged plant. Feeding by beet armyworm larvae (*Spodoptera exigua*) on the lower leaves of cotton plants triggers the systemic release of six readily detectable terpenes and hexenyl acetate, a product of the lipoxygenase pathway. Labelling with $^{13}CO_2$ established that the terpenes released systemically after herbivore damage are synthesized *de novo* at or near the site of emission. Hexenyl acetate rapidly incorporated ^{13}C into the acetate but not into the hexenyl moiety within our pulse labelling interval. Thus, activation of the lipoxygenase pathway in undamaged leaves of insect damaged plants is indicated.

INTRODUCTION

Plants respond to insect feeding damage by releasing volatile compounds that attract natural enemies of insect herbivores [1–3]. Blends of volatile terpenoids and other compounds that are released [4] allow insect parasitoids, such as parasitic wasps [3], and predators [5] to distinguish between infested and non-infested plants and thus aid in location of hosts or prey. These phytodistress signals, which result in an active interaction between herbivore-damaged plants and a third trophic level, have been described for several plant species. Examples include lima beans that produce volatiles that attract predatory mites when damaged by spider mites [5] or corn plants that produce volatiles that attract the hymenopterous larval parasitoids *Cotesia marginiventris* when under attack by beet armyworm caterpillars [6, 7].

A large number of compounds have been identified in the collection of head space volatiles from different plant species both with and without insect feeding. Included in the list are fatty acid derived aldehydes and alcohols [8], terpenes derived from mevalonic acid, aromatic metabolites such as indole and methyl salicylate derived from shikimic acid [9], and a variety of hydrocarbons released during periods of high temperatures [10]. Volatiles released by corn and cotton in response to beet armyworm damage induce cyclic and acyclic terpenes, a variety of lipoxygenase products, and indole [7, 11]. Clearly, more than one biosynthetic pathway is responsible for this phytochemical release [12].

Loughrin *et al.* [13] suggested that in cotton some monoterpenes and sesquiterpenes are stored in the leaves, providing built-in protection against invading organisms, while others are produced and released with the onset of feeding damage. The constitutive compounds are identified by their almost immediate release after feeding begins, their relatively constant release over time, and their rapid wane after feeding ceases. It has been proposed that such metabolites are synthesized in specialized cells such as mesophyll pigment glands which are embedded in the leaves of cotton [14, 15]. These stored volatiles can be released by simply breaking the glands as a result of herbivore feeding. In contrast, the induced compounds show a delay between the time feeding starts and the release of volatiles. For example, in cotton this delay of compound release is between 12 and 24 hr after herbivore feeding begins [13]. In addition, these induced compounds show a diurnal cycling of release which continues after herbivore feeding has ceased. In Fig. 1, the bold type-face compounds listed are those compounds from cotton that are induced with beet armyworm feeding on the leaves whereas the standard type-face compounds are those compounds that are released from storage, presumably in the glands of the leaves.

* This paper is dedicated to Clarence (Bud) Ryan on the occasion of his sixty-fifth birthday.

† Author to whom correspondence should be addressed.

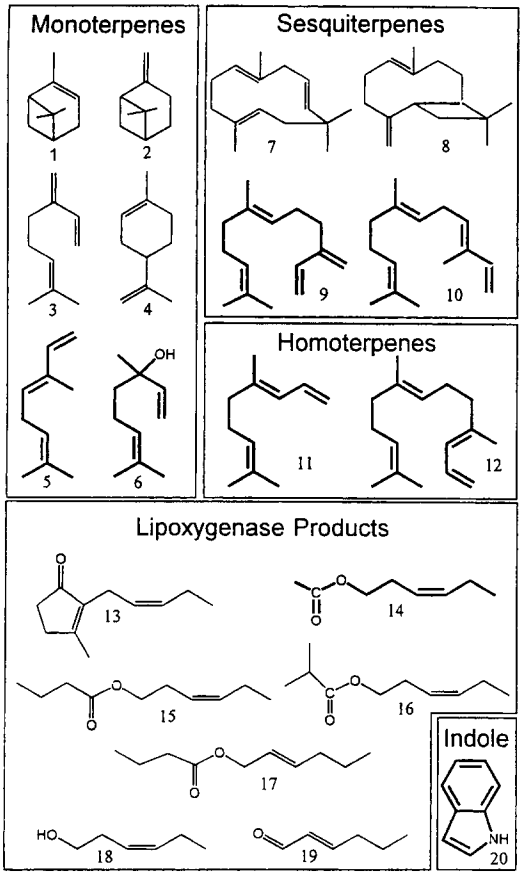

Fig. 1. Compounds detected in head space volatiles collected from cotton plants damaged by beet armyworm larvae feeding on leaves. Standard typeface compounds are released from storage and incude: α-pinene **1**, β-pinene **2**, myrcene **3**, limonene **4**, α-humulene **7**, β-caryophyllene **8**, jasmone **13**, (*Z*)-3-hexenyl butyrate **15**, (*Z*)-3-hexenyl isobutyrate **16**, (*E*)-2-hexenyl butyrate **17**, (*Z*)-3-hexen-1-ol **18** and (*E*)-2-hexenal **19**. Bold typeface compounds are biosynthesized *de novo* just prior to release and include: (*E*)-β-ocimene **5**, linalool **6**, (*E,E*)-α-farnesene **9**, (*E*)-β-farnesene **10**, (*E*)-4,8-dimethyl-1,3,7-nonatriene **11**, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene **12**, (*Z*)-3-hexenyl acetate **14**, and indole **20**.

We have recently demonstrated in labelling studies with $^{13}CO_2$ that, in cotton, several of the volatile terpenes induced by insect herbivore damage are synthesized *de novo*, with little or no release of these compounds from storage [16, 17]. In addition to several of the acyclic terpenes and sesquiterpenes being labelled, indole, a product of the shikimic acid pathway, is also synthesized *de novo* in response to insect damage. These induced volatiles incorporate larger amounts of ^{13}C and are produced and released in greater quantities as a result of beet armyworm feeding or exogenous application of beet armyworm oral secretion to mechanically damaged leaf tissue than with mechanical damage alone. In contrast, the constitutive compounds incorporate almost undetectable amounts of ^{13}C and their release seemed to depend solely on the amount of damage caused by either mechanical injury or insect feeding. This provides strong evidence that in this system *de novo* biosynthesis of volatiles is triggered by an elicitor from the oral secretion of the feeding caterpillar.

In fact, two different types of insect-derived volatile elicitors have recently been reported. One is a β-glucosidase identified from the saliva of *Pieris brassicae* larvae which feed on Brussels sprouts and induces the release of volatiles [18]. In agreement with the activity of this protein, the same laboratory group has observed the release of volatiles from Brussels sprouts when commercially purified almond β-glucosidase is exogenously applied to mechanically wounded leaves. The second, an elicitor from the oral secretions of beet armyworms, *Spodoptera exigua* Hübner, has been identified as *N*-(17-hydroxylinolenoyl)-(L)glutamine [19]. Biological activity of the synthesized compound confirmed the chemical structure. This compound, called volicitin, when supplied in amounts of about 300 pmol to corn seedlings through cut stems, or by application to a mechanically damaged site, induces the release of volatiles specific to insect feeding from all the leaves of the plant [19, 20]. The similarities between the structure of volicitin and the substrates of the octadecanoid signalling pathway [21] in plants are intriguing. In the plant systems studied thus far, biosynthesis and release of volatile compounds appear to be induced by jasmonic acid, which is derived from linolenic acid [22]. Jasmonates are also known to stimulate numerous other physiological and defensive processes in plants [22, 23]. Furthermore, the amino acid conjugates of jasmonic acid are involved in physiological and developmental processes in many plants [22, 24]. Thus, the structure of volicitin, an octadecenoate conjugated to an amino acid, suggests a chemical link between herbivores and the amplification of the octadecanoid signalling pathway in insect-damaged plants.

In addition to the release of volatiles at the site of herbivore feeding, analysis of volatile emissions from unharmed leaves of insect damaged plants has established that there is a systemic response. In both corn [25] and cotton [26], leaves distal to the site of herbivore feeding showed an increase in the release of volatiles. The chemical blend of volatiles from undamaged leaves differs from the volatiles collected from the entire plant [26]. Except for (*Z*)-3-hexenyl acetate, the hexenals and hexenols of the lipoxygenase pathway, which are released from freshly cut or damaged tissue, are not emitted systemically. In cotton, some of the monoterpenes and sesquiterpenes, as well as indole and isomeric hexenyl butyrates and 2-methyl butyrates, are only released from damaged leaves [26]. The terpenoids that are synthesized *de novo* in cotton leaves in response to herbivore damage [17] are also released systemically from undamaged leaves of an herbivore injured plant. The terpenoids that did not incorporate ^{13}C when damaged cotton plants were exposed to $^{13}CO_2$ were not released systemically.

These results raised the question of whether the

compounds that are released systemically are synthesized *de novo* in the leaves from which they are released, or whether they are synthesized in the damaged leaves and transported to the undamaged leaves. To answer this question, we conducted experiments in which the undamaged leaves in the upper portion of the plant were exposed to air containing $^{13}CO_2$ and volatiles were collected and analysed by gas chromatography-mass spectroscopy.

RESULTS AND DISCUSSION

Systemic release of volatiles

Beet armyworm larvae were caged and allowed to feed on the lower leaves of a plant at the start of the experiment and volatiles were collected and analysed from the top undamaged leaves of the plant beginning 45 hr later. Insect damage was < 10% of the total leaf area of the plant. Seven compounds were released in greater quantities from the top undamaged leaves of injured plants than from the top leaves of undamaged control plants (Fig. 2). The systemically released compounds were the same compounds previously found to be induced by caterpillar feeding (Fig. 1) [16] and to be synthesized *de novo* by caterpillar damaged plants [17]. The only exception was indole, which was not found in the systemically released volatiles. These results are consistent with those of Röse *et al.* [26].

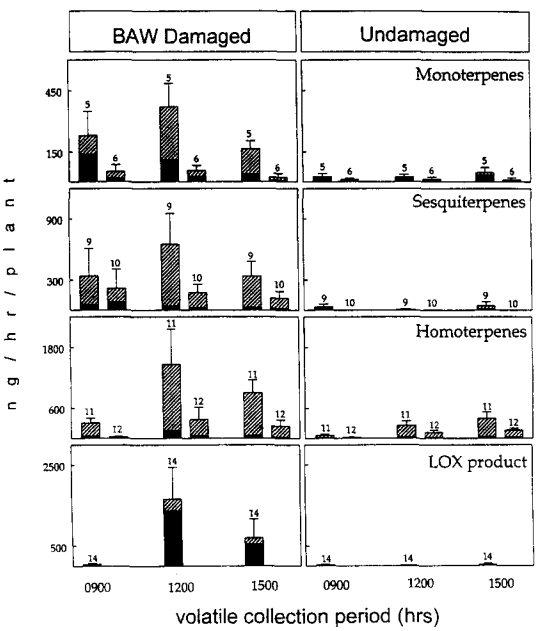

Fig. 2. Volatiles released systemically from cotton plants with (BAW damaged) and without (undamaged) beet armyworm larvae feeding on the lower leaves. Hatched areas represent ^{13}C-labelled product and solid areas represent non-labelled product. See Fig. 1 for numbering of compounds; mean (±s.e.m.) volatile release is shown with each data point ($n = 3$).

De novo *biosynthesis of systemic volatiles*

Highly enriched $^{13}CO_2$ at atmospheric concentrations was added to the chambers containing the top undamaged leaves of both the damaged and the control plant during the same time that volatiles were collected. As indicated in Fig. 2, a high level of ^{13}C was rapidly incorporated into all the systemically released volatiles. As a second control, $^{13}CO_2$ was passed over the bottom, damaged leaves of a plant while volatiles were collected from the top, undamaged leaves, over which filtered ambient air was being drawn. In this case, the mass spectra of the systemic volatile compounds were not different from synthetic standards, indicating no incorporation of the label (data not shown). These results clearly indicate that the volatiles released systemically in response to herbivore damage are synthesized *de novo* in the undamaged leaves from which they are released and distal from the site of damage. Therefore, a signal is transmitted by either volicitin [19] or another messenger from the site of damage to distal, undamaged leaves to trigger *de novo* biosynthesis and release of volatile compounds.

Labelling the top undamaged portion of the cotton plant with $^{13}CO_2$ in tandem with GC-chemical ionization mass spectrometric analysis of the systemic volatiles released provided an account of the compounds synthesized at or near the site of release and not at the site of wounding. It also gave a detailed picture of the distribution of the label for each compound. The normal distribution pattern for the labelled carbon incorporation indicated by the $[M+1]^+$ up to $[M+n]^+$, with n being the number of carbons in the molecule, suggests that there is random labelling throughout the molecules for each of the terpenes [for example see Fig. 3(D)]. In contrast, for (*Z*)-3-hexenyl acetate the M+2 and M+3 ions corresponding to one and two ^{13}C atoms added to the molecule are the predominantly labelled molecular ions [Fig. 3(B)]. This suggests that there is site specific incorporation of the ^{13}C label with the acetate portion becoming heavily labelled while the hexenyl portion remains unlabelled. In fact the *m/z* 83 through 83+*n* associated with the hexenyl fragment is the same pattern in the labelled and unlabelled molecule, indicating no detectable enrichment of the hexenyl portion of the molecule. These results indicate that the lipoxygenase pathway is activated in the undamaged leaves distal from the site of feeding damage, but that the linolenic acid is obtained from storage rather than being synthesized *de novo*.

The hexenals and hexenols, products of the action of lipoxygenase enzymes on linolenic acid, as are the defense signal molecules jasmonic acid and methyl jasmonate [8], are produced and released from damaged regions of leaves. Perhaps they cannot be released from undamaged leaves in significant amounts unless these six-carbon compounds are converted into the acetate form. This could explain the very high levels of partially labelled (*Z*)-3-hexenyl

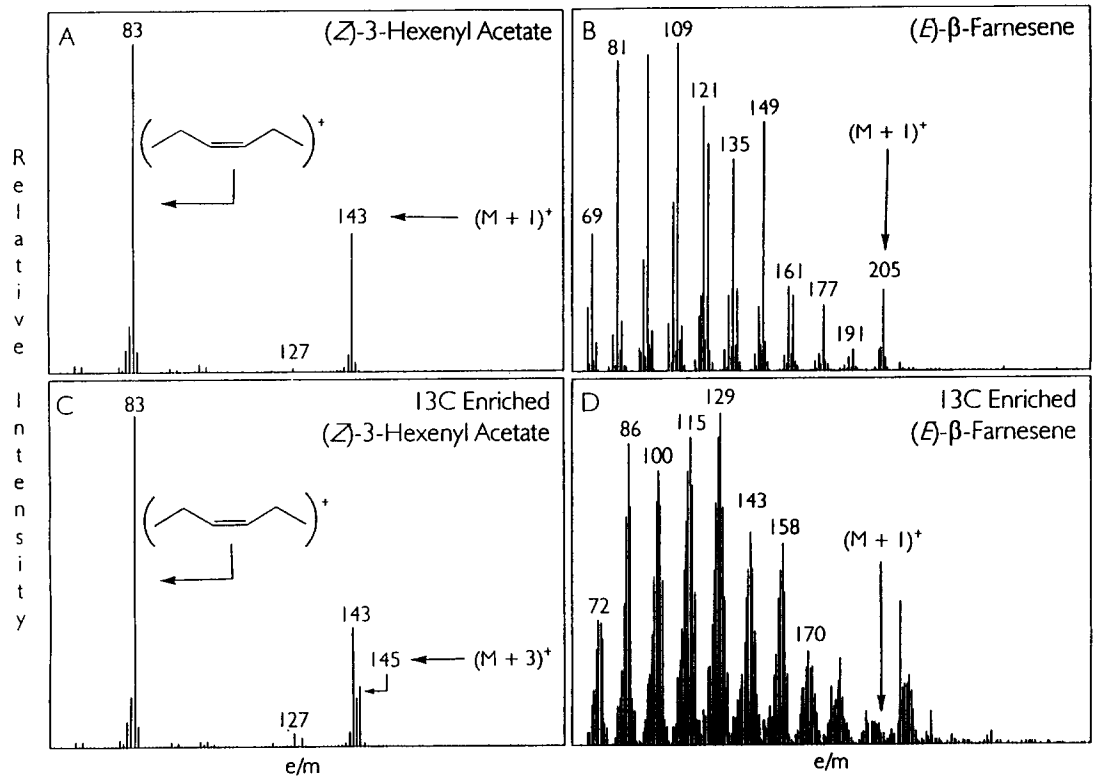

Fig. 3. Chemical ionization mass spectra of (*Z*)-3-hexenyl acetate (panels A and C) and *E*-β-farnesene (panels B and D) released systemically from undamaged upper leaves with 48-hr of beet armyworm feeding on lower cotton leaves. In the top panels spectra represent volatiles collected from plants in chambers with filtered ambient air, whereas in the bottom panels, spectra were obtained on volatiles collected from plants in a $^{13}CO_2$ enriched chamber.

acetate released systemically from unwounded leaves of insect damaged plants compared to undamaged controls. The activation of the lipoxygenase pathway in the undamaged leaves suggests a mechanism analogous to that proposed by Farmer and Ryan [21], wherein a mobile signal such as systemin can transmit information from the damaged site to the distal leaves triggering the lipoxygenase pathway and resulting in a cascade of signals activating several defense responses in the plant. If in fact there is such an up regulation of the lipoxygenase pathway, it will be interesting to examine the difference between insect damaged plants and undamaged plants in the pool of chemical intermediates in the undamaged leaves. Also of interest is the possibility of induced biochemical regulation of an enzyme such as acetyl transferase in the formation of (*Z*)-3-hexenyl acetate.

EXPERIMENTAL

Insects and plant. Cotton plants (*Gossypium hirsutum* L., var. Deltapine Acala 90) grown from seeds were maintained in an insect-free greenhouse. Greenhouse temp. was maintained at $29 \pm 4°$ with a relative humidity minimum of $40 \pm 5\%$ in the late afternoon and a maximum of $95 \pm 5\%$ in the early morning. Since plants were grown during the winter, 400 W high pressure sodium lamps were used to supplement natural light with a 16 hr light/8 hr dark photoperiod. Plants were grown in 16 cm diameter pots containing Metromix 300 potting soil (Scotts–Sierra Hort. Co.; Marysville, OH). Six-week-old plants that were 25–30 cm tall and had not set flower buds were used in labelling studies. Beet armyworms (*Spodoptera exigua* Hübner) were reared on artificial diet in this laboratory by the method of King and Leppla [27]. Fourth-instar caterpillars were starved for 7 hr prior to being placed on plants.

Plant wounding and volatile collection. All experiments were conducted in the same greenhouse in which the plants were grown. Two larvae per leaf were caged on the bottom two leaves and on one of the cotyledons at the start of the experiment (1200 hr, day 1) and were allowed to feed continuously throughout the experiment. The leaves on which the larvae fed were outside the volatile collection apparatus (see later section). Caterpillars were removed at the end of the damage treatment and leaves were photocopied the following morning. Total leaf area and insect-damaged portions were measured by scanning a photocopy of the leaves (Sigma Scan, Jandel Sci.; Sausalito, CA).

Volatile chemicals were collected in the greenhouse from intact cotton plants on the third day. Three-hour volatile samples were taken at times when partially damaged plants emitted a maximum of volatiles (0900

to 1200, 1200 to 1500 and 1500 to 1800 hr) [13]. Volatiles were collected from the undamaged upper leaves while caterpillars fed on the lower leaves of the same plant. Volatiles were also collected simultaneously from the upper leaves of an undamaged control plant under identical conditions and from the upper undamaged leaves of control plant with $^{13}CO_2$ passed over insect-damaged leaves below the chamber at a flow rate of 1 l min^{-1} (see labelling section).

To collect volatiles from the undamaged parts of a caterpillar-damaged plant and control plants, the upper leaves of each plant were enclosed in separate 'guillotine' volatile collection chambers that were part of an automated volatile collection system previously described [26, 28, 29]. This system allows collection of volatiles from the upper portion of the cotton plants while completely isolating the lower section of the plant where caterpillars were feeding in the systemic treatment. Volatiles were collected on Super Q adsorbent traps with 50% (1 l min^{-1}) of the air passing over the undamaged upper leaves of the plant pulled through a trap. The remainder of the air escaped through the base of the volatile collection chamber.

Analysis and identification of volatile compounds. Compounds were eluted from the adsorbent traps with 150 μl of CH_2Cl_2; 400 ng each of *n*-octane and nonyl acetate were added as int. standards and 1 μl aliquots were analysed by capillary GC on a 50 m × 0.25 mm (i.d.) fused-silica column with a 0.25 μm thick bonded methyl silicone stationary phase (Quadrex, New Haven, CT). Injections were made in the splitless mode for 30 s, and the gas chromatograph was operated under the following conditions: injector 220°, detector 220°, column oven 60° for 2 min, then programmed at 4° min^{-1} to 180°, He carrier gas linear flow velocity 19 cm sec^{-1}. For identification of compounds, selected samples were also analysed by GC-MS on a Finnigan MAT ITS40 (ion trap) mass spectrometer interfaced to a Varian model 3400 gas chromatograph and operated in the electron impact mode. Injections were made *via* a septum-equipped programmable injector (SPI) held at 40° for 0.25 min then programmed at 170° min^{-1} to 270° onto a 30 m × 0.25 mm (i.d.) fused-silica column with 0.25 μm thick bonded 5% phenyl methyl silicone (DB-5MS; J and W Scientific) held at 40° for 5 min, then programmed at 5° min^{-1} to 260°; He carrier gas linear flow velocity was 19 cm sec^{-1}. Source temp. was adjusted to 120 ± 20° to optimize the molecular ion abundance. Components of the plant volatile emission were identified by comparison of GC R_ts with those of authentic standards on both capillary columns and by comparison of mass spectra with spectra of an Environmental Protection Agency/National Institutes of Health data base.

In vivo *labelling.* Synthetic premixed air (Cambridge Isotope Laboratories; Andover, MA and Airco; Riverton, NJ) which contained 360 ppm carbon dioxide (^{13}C 99%), 20.7% oxygen and a balance of N_2 was introduced into the volatile collection apparatus by flushing the chamber at 10 l min^{-1} for 2 min and then reducing the flow to 2 l min^{-1}. The same purging procedure was followed to switch back to atmospheric air. Labelled carbon dioxide air was introduced into the chambers at 0900 hr on day 3 for a 9 hr period and volatiles were collected in 3 hr intervals.

Compounds were quantified *via* flame ionization detection using the peak area of nonyl acetate (400 ng) added as an int. standard. To determine the amount of ^{13}C incorporated into each compound, all samples were analysed by the Finnigan MAT ITS40 (ion trap) mass spectrometer as previously described, except that the ion trap was operated in the chemical ionization mode with isobutane as the reagent gas (GS-CIMS). Selected mass ions were quantitated *via* computer software analysis. By summing the intensities of the $[M+3]^+$ through $[M+n]^+$ (n = number of carbons in the molecule) ions and comparing this with the intensity of the $[M-1]^+$ through the $[M+2]^+$ ion signals, the contribution of ions associated with enriched and unenriched molecules and in turn the $^{13}C/^{12}C$ ratio for each compound was determined. Using all the ion peaks associated with the naturally enriched volatile products insured that enrichment levels were not over estimated. Because the molecular ion for the monoterpene linalool with its hydroxyl functional group did not appear, the $[M+1]^+$-18 ion was used to calculate the ratio between enriched and unenriched molecules.

REFERENCES

1. Tumlinson, J. H., Lewis, W. J. and Vet, L. E. M., *Scientific American*, 1993, **268**, 100.
2. Stowe, M. K., Turlings, T. C. J., Loughrin, J. H., Lewis, W. J. and Tumlinson, J. H., *The Proceedings of the National Academy of Sciences U.S.A.*, 1995, **92**, 23.
3. Turlings, T. C. J., Loughrin, J. H., McCall, P. J., Rose, U. S., Lewis, W. J. and Tumlinson, J. H., *The Proceedings of the National Academy of Sciences U.S.A.*, 1995, **92**, 4169.
4. Turlings, T. C. J., Tumlinson, J. H. and Lewis, W. J., *Science*, 1990, **250**, 1251.
5. Dicke, M., van Baarlen, P., Wessels, R. and Dijkman, H., *The Journal of Chemical Ecology*, 1993, **19**, 581.
6. Turlings, T. C. J., Tumlinson, J. H., Heath, R. R., Proveaux, A. T. and Doolittle, R. E., *The Journal of Chemical Ecology*, 1991, **17**, 2235.
7. Turlings, T. C. J. and Tumlinson, J. H., *The Florida Entomologist*, 1991, **7**, 42.
8. Croft, K. P., Juttner, R. and Slusarenko, A. J., *Plant Physiology*, 1993, **101**, 13.
9. Mann, J., *Secondary Metabolism.* Clarendon Press, Oxford, England, 1987.
10. Sharkey, T. D. and Singsaas, E. L., *Nature*, 1995, **374**, 769.
11. McCall, P. J., Turlings, T. C. J., Loughrin, J.,

Proveaux, A. T. and Tumlinson, J. H., *The Journal of Chemical Ecology*, 1994, **20**, 3039.
12. Paré, P. W. and Tumlinson, J. H., *The Florida Entomologist*, 1996, **79**, 93.
13. Loughrin, J. H., Manukian, A., Heath, R. R., Turlings, T. C. J. and Tumlinson, J. H., *The Proceedings of the National Academy of Sciences U.S.A.*, 1994, **91**, 11836.
14. Gershenzon, J., Maffei, M. and Croteau, R., *Plant Physiology*, 1989, **89**, 1351.
15. Croteau, R. and Johnson, M. A., in *Biology and Chemistry of Plant Trichomes*, ed. E. Rodriguez, P. L. Healy and I. Mehta. Plenum Publishing, New York, 1984, p. 133.
16. Paré, P. W. and Tumlinson, J. H., *Plant Physiology*, 1997, **114**, 1161.
17. Paré, P. W. and Tumlinson, J. H., *Nature*, 1997, **385**, 30.
18. Mattiacci, L., Dicke, M. and Posthumus, M. A., *The Proceedings of the National Academy of Sciences U.S.A.*, 1995, **92**, 2036.
19. Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H. and Tumlinson, J. H., *Science*, 1997, **276**, 945.
20. Turlings, T. C. J., McCall, P. J., Alborn, H. T. and Tumlinson, J. H., *The Journal of Chemical Ecology*, 1993, **19**, 411.
21. Farmer, E. E. and Ryan, C. A., *The Plant Cell*, 1992, **4**, 129.
22. Krumm, T., Bandemer, K. and Boland, W., *FEBS Letters*, 1995, **377**, 523.
23. Farmer, E. E. and Ryan, C. A., *Trends in Cell Biology*, 1992, **2**, 236.
24. Kramell R., Atzorn, R., Schneider, G., Miersch, O., Bruckner, C., Schmidt, J., Sembdner, G. and Parthier, B., *The Journal of Plant Growth Regulation*, 1995, **14**, 29.
25. Turlings, T. C. J. and Tumlinson, J. H., *The Proceedings of the National Academy of Sciences U.S.A.*, 1992, **89**, 8399.
26. Röse, U. S. R., Manukian, A., Heath, R. R. and Tumlinson, J. H., *Plant Physiology*, 1996, **111**, 487.
27. King, E. G. and Leppla, N. C., *Advances and Challenges in Insect Rearing*. U.S. Government Printing Service, Washington, D.C., 1984.
28. Heath, R. R. and Manukian, A.,*The Journal of Chemical Ecology*, 1992, **18**, 1209.
29. Manukian, A. and Heath, R. R., *Scientific Computing and Automation*, 1993, **9**, 27.